IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Glazer et al.	Group Art Unit: 1653
Serial No. Not yet assigned	Examiner: Kam, Chih Min
Filed: Herewith	Attorney Docket No. B00-016-3
For: *Multifunctional Recombinant Phycobiliprotein-Based Fluorescent Constructs and Phycobilisome Display*	Date: July 10, 2003 This is a continuation of USSN 09/469,194, filed 12/21/99, now allowed, having the same title and inventors

TRANSMITTAL LETTER

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

This is a continuing application of allowed, copending Serial No. 09/469,194, filed December 21, 1999, having the same title and inventors. A second continuing application of allowed, copending Serial No. 09/469,194, filed December 21, 1999, having the same title and inventors is also being filed today.

The enclosed specification is identical to the parent (Serial No. 09/469,194) except for addition of cross-related application information on p.1, further characterizing on p.4, the prior art Glazer et al (1994) reference (enclosed) as disclosing exploiting intermolecular energy transfer by functionally coupling fluorescent phycobiliprotein tags with other fluorescent tags, such as cyanine dyes (see, Glazer, 1994 at p.109), and inserting a new set of claims.

The claims are directed to compositions comprising the fusion proteins claimed in allowed, copending 09/469,194, but further requiring that they provide a fluorescent tag. Support for the recited luminescent properties is found, inter alia, on p.5, lines 7-17. Support for the recited cell types is found, inter alia, at p.4, lines 14-15. That oligomeric phycobiliproteins comprising the fusion proteins provide specific labeling reagents, tags and phycobiliprotein-labeled fluorescent reagents is found, inter alia, on p.2, lines 4-9; p.2, lines 12-13; p.2, line 28-29; and p.6, line 20.

This submission introduces no new matter.

Respectfully submitted,
SCIENCE & TECHNOLOGY LAW GROUP

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Phycobiliproteins – a family of valuable, widely used fluorophores

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Abstract

Phycobiliproteins are brilliantly colored, highly fluorescent components of the photosynthetic light-harvesting antenna complexes of cyanobacteria (blue-green algae), red algae and cryptomonads. These proteins carry covalently attached linear tetrapyrrole pigments related structurally to biliverdin. Phycobiliproteins, purified from certain organisms, are isolated as either trimers, $(\alpha\beta)_3$, of approximately M_r 110–120×10^3 (e.g., allophycocyanins), or hexamers, $(\alpha\beta)_6\gamma$, of about M_r 250×10^3 (certain phycoerythrins). Three phycobiliproteins R-phycoerythrin, B-phycoerythrin, and allophycocyanin serve as valuable fluorescent tags with numerous applications in flow cytometry, fluorescence activated cell sorting, histochemistry and, to a limited degree, in immunoassay and detection of reactive oxygen species. These applications exploit the unique physical and spectroscopic properties of phycobiliproteins.

Introduction

The blue-green and red colors of cyanobacterial and red algal cells are diagnostic of the major components of the light-harvesting antenna complexes in these organisms – the phycobiliproteins (Glazer, 1981). In cyanobacteria cultured at low light intensities in the presence of abundant nutrients, the phycobiliproteins can make up as much as 40% of the protein of the cell. In many red algae, grown under similar conditions, phycoerythrin, their most abundant phycobiliprotein, is the major soluble protein in the cell.

In cyanobacteria and red algae, the phycobiliproteins are components of a macromolecular light-harvesting complex, the phycobilisome, which in intact cells is associated with the cytoplasmic surface of the thylakoid membrane (Gantt, 1980). When the cells are broken in dilute buffers at near-neutral pH, the phycobilisomes dissociate into a multiplicity of intensely colored, highly fluorescent, water-soluble subcomplexes. Purified phycobiliproteins represent the most stable of such individual subcomplexes. Phycobiliproteins are readily purified by conventional techniques of protein fractionation and are named according to their spectroscopic properties.

In the cryptomonads, only a single type of phycobiliprotein is present in any given strain. The subunit structure of purified native cryptomonad biliproteins is $\alpha\alpha'\beta_2$ with a M_r about 50×10^3 (Mörschel & Wehrmeyer, 1975; Wedemayer, 1991).

The colors of the phycobiliproteins arise from the presence of covalently attached prosthetic groups – bilins. The bilins are linear tetrapyrroles derived biosynthetically from heme via biliverdin (Beale & Cornejo, 1991). The structures of four different isomeric bilins, those found to date in the phycobiliproteins of cyanobacteria and red algae, are shown in Fig. 1. Several additional tetrapyrroles have been recently found in cryptomonad phycobiliproteins (Wedemayer *et al.*, 1991; Wemmer *et al.*, 1993). The visible absorption spectra of individual phycobiliproteins arise from the particular bilins attached to the protein as modulated in a major way by the conformation, environment, and interchromophore interactions dependent on the native protein structure (Glazer, 1986). The latter factor is particularly noticeable with respect to phycobiliprotein fluorescence: native phycobilipro-

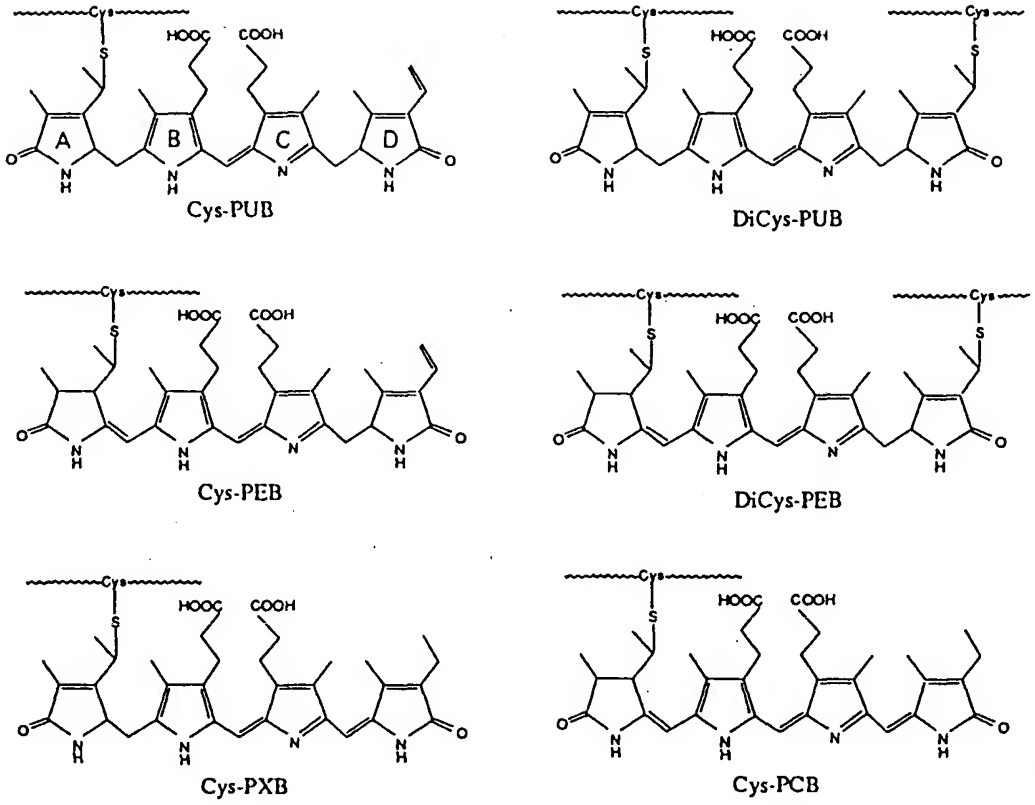

Fig. 1. Structures of the cysteine-linked bilins found as prosthetic groups on cyanobacterial or red algal phycobiliproteins. Cys-PCB, Cys-PXB, Cys-PEB, and Cys PUB designate phycocyanobilin, phycobiliviolin, phycoerythrobilin and phycourobilin, respectively, each linked through a single thioether bond at C3′ to a cysteinyl residue. DiCys-PEB and DiCysPUB designate phycoerythrobilin and phycourobilin, respectively, each linked through two thioether bonds at C3′ and C18′. Within native phycobiliproteins, the absorption maxima of the bilins are at the following approximate wavelengths: PCB 620–650 nm, PXB 568 nm, PEB 540–565, PUB 490 nm.

teins are highly fluorescent; denatured ones are either non-fluorescent or very weakly so.

Physical and spectroscopic properties of certain phycoerythrins and allophycocyanin

The phycobiliproteins were introduced as a novel class of fluorescent tags in 1982 (Oi *et al.*, 1982; Glazer & Stryer, 1984) and immediately came into widespread use in many diagnostic clinical assays, in histochemistry, and in diverse research applications. A continuing steady expansion in the use of phycobiliproteins has occurred since that time (Glazer & Stryer, 1990).

Some of the advantages of phycobiliproteins as fluorescent tags over simple organic fluorescent dyes are clarified through a discussion of the physical and spectroscopic properties of the phycobiliproteins. This discussion will be confined to the phycoerythrins and allophycocyanin. These are the most widely used of the phycobiliproteins, and the points raised in describing these proteins are broadly pertinent to other phycobiliproteins as well.

Phycoerythrins

Phycoerythrins are the most abundant phycobiliproteins in many red algae and in some unicellular cyanobacteria. Phycoerythrins purified from red algae are stable hexameric disk-shaped complexes, 12 nm in diameter and 6 nm high, with the subunit structure $(\alpha\beta)_6\gamma$ and a M_r 250×10^3. Each hexamer carries 34 bilins. The absorption spectra of these proteins show strong peaks between 480 and 570 nm. They fluoresce

strongly at about 580 nm. The majority of red algal phycoerythrins falls into two major classes - B- and R-phycoerythrins (Glazer *et al.*, 1982; Honsell *et al.*, 1984). B-phycoerythrins carry 32 phycoerythrobilins (Fig. 1, PEBs) which absorb maximally at about 550 nm and 2 phycourobilins (Fig. 1; PUBs) which absorb maximally at 490 nm (Lundell *et al.*, 1984). Consequently, the spectra of these proteins have strong absorption maxima between 540 and 567 nm, but only a shoulder at 490 nm. R-phycoerythrins carry 25 PEBs and 9 PUBs (Klotz & Glazer, 1985). These proteins have strong absorption maxima both at 490 nm and between 540 and 567 nm (Fig. 2A). Both B- and R-phycoerythrin conjugates are used extensively in cell sorting and cell analyses.

Allophycocyanin

Purified allophycocyanin is a disk-shaped trimer, $(\alpha\beta)_3$, 11 nm in diameter and 3 nm high, of M_r about 110×10^3. This protein carries a phycocyanobilin (PCB) on each subunit, six per trimer. Allophycocyanin has a sharp absorption maximum at 650 nm and an emission maximum at 660 nm. Allophycocyanin dissociates to the monomer, $\alpha\beta$, at low concentrations (e.g. 100 ng ml^{-1}) with a large decrease in maximum absorbance and a blue shift in the maximum to 620 nm, with a corresponding shift and decrease in the fluorescence emission. This dissociation can be prevented by limited crosslinking of the allophycocyanin trimer with a water-soluble crosslinker, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, without much alteration in spectroscopic properties (Ong & Glazer, 1985). The crosslinked allophycocyanin trimer does not dissociate on dilution and is much more thermostable than the unmodified trimer (Yeh *et al.*, 1987).

Properties of phycobiliproteins relevant to their use as fluorescent tags

Isoelectric points

The isoelectric points of phycobiliproteins range from 4.7–5.3 (Glazer, 1981). The proteins are negatively charged at physiological pH values and cell surfaces are negatively charged. Consequently, there is minimal non-specific binding of native phycobiliproteins (or their conjugates) to intact cells. This is a very important factor in the use of phycobiliprotein conjugates in fluorescence-activated cell sorting or in flow cyto-

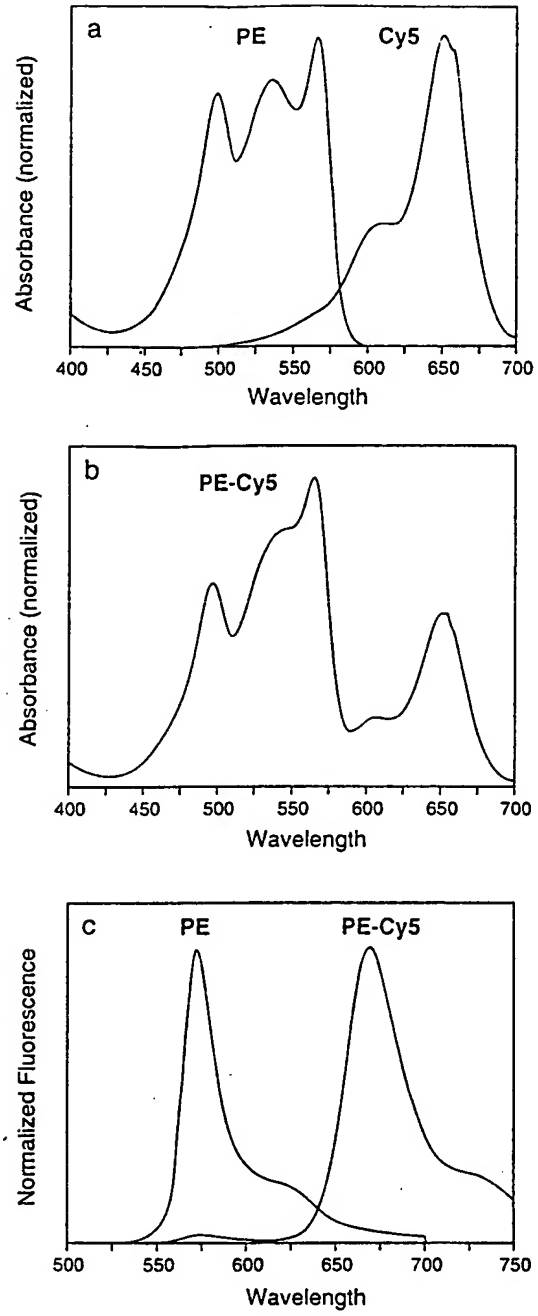

Fig. 2. Spectroscopic properties of R-phycoerythrin (PE) and of a R-phycoerythrin conjugate with the cyanine dye Cy5.18 (Cy5; see Fig. 3). Panel a, absorption spectrum of PE and of Cy5. Panel b, absorption spectrum of a PE-Cy5 conjugate containing on average 5.2 Cy5 per PE. Panel c, fluorescence emission from PE and from the PE-Cy5 conjugate seen on excitation at 488 nm. Note that the energy transfer from PE to Cy5 in the PE-Cy5 conjugate is very efficient. The emission from the PE-Cy5 conjugate emanates almost entirely from Cy5, with only a trace of residual emission from PE. (Data from Waggoner *et al.*, 1993.)

metric analyses.

In contrast, when many molecules of a dye, such as fluorescein, are conjugated to a single antibody molecule, or some other macromolecule with biological recognition specificity, non-specific binding to cells frequently occurs.

Synthesis of conjugates

The phycobiliproteins are highly water-soluble and show no change in spectroscopic or other physical properties on storage in aqueous solution for long periods of time. These high-molecular-weight proteins have numerous surface functional groups (ε-NH_2 or β- and γ-carboxyl) per molecule. These can be readily coupled to a variety of small molecules (e.g. biotin, digoxigenin) or proteins (e.g. monoclonal antibodies, avidin, streptavidin). As long as only a small number (3 to 6) of phycobiliprotein side-chains are modified per molecule, there is little alteration in the physical or spectroscopic properties of the phycobiliprotein.

Location of the bilins within the three-dimensional structure of phycobiliproteins

Crystal structures of three phycobiliproteins trimeric and hexameric C-phycocyanin (Schirmer *et al.*, 1987; Duerring *et al.*, 1991), trimeric phycoerythrocyanin (Duerring *et al.*, 1990), and hexameric B-phycoerythrin (Ficner *et al.*, 1992) have been solved at high resolution. The bilin chromophores are in all instances held in extended conformations and have numerous interactions with the polypeptide. The bilins are largely shielded from solvent, a fact in keeping with the observation that the fluorescence of native phycobiliproteins is not subject to significant collisional quenching by small molecule solutes.

Molar absorption coefficients (ε_M) and fluorescence quantum yields (Φ_F)

An excellent introductory treatment of photophysics relevant to the assessment of the spectroscopic properties of various fluorophores is provided by Clayton (1970). Briefly, the phycobiliproteins have very high molar absorption coefficients. For example, B-phycoerythrin has an ε_M (545 nm) of 2.41×10^6 $M^{-1}cm^{-1}$ per hexamer (Glazer & Hixson, 1977), and allophycocyanin has an ε_M (650 nm) of $6.96\times10^5 M^{-1}$ cm^{-1} per trimer (Cohen-Bazire *et al.*, 1977). The fluorescence quantum yield for B-phycoerythrin is 0.98 (λ^F_{max} 575 nm) and that for allophycocyanin is 0.68 (λ^F_{max} 660 nm) (Grabowski & Gantt, 1978). These values should be compared to those for two exceptionally good, widely used synthetic fluorescent molecules – fluorescein and the cyanine dye Cy5.18. For fluorescein in aqueous solution, ε_M (490 nm) is 8×10^4 M^{-1} cm^{-1} and Φ_F is 0.9 (λ^F_{max} 530 nm). For Cy5.18, ε_M (650 nm) is 2.5×10^5 M^{-1} cm^{-1} and Φ_F is 0.27 (λ^F_{max} 667 nm) (Fig. 3; Waggoner *et al.*, 1993).

For equimolar solutions of fluorescein and B-phycoerythrin hexamer excited at the 488 nm argon ion laser line, the fluorescence from the phycoerythrin solution is 14.5 times more intense than that from the fluorescein solution (Oi *et al.*, 1982). Similarly, for equimolar solutions of a Cy5.18 alkylamine derivative (Fig. 3) and allophycocyanin trimer excited at 633 nm with a He-Ne laser, the fluorescence intensity from the allophycocyanin solution is about 7 times greater.

Stokes shifts

The displacement of the fluorescence spectrum relative to the absorption spectrum is called the Stokes shift. The Stokes shift for strongly absorbing organic dyes is in general $\leq$ 30 nm. In contrast, with appropriate choice of excitation wavelength, the Stokes shifts attainable with phycobiliproteins can be very large, 80 nm or greater. As noted above, the phycobiliproteins contain numerous bilin chromophores that may differ in their chemical structure (Fig. 1), and that do differ in their protein environment. As a consequence, the absorption spectra of the phycobiliproteins are broad, allowing efficient excitation over a wide range of wavelengths. The excitation quanta absorbed by the various bilins in a phycobiliprotein are transferred by radiationless processes to acceptor bilins whose absorption bands lie at the red edge of the phycobiliprotein absorption spectrum (Ong & Glazer, 1991; e.g., see Fig. 2 in Glazer & Stryer, 1984). For example, excitation of R-phycoerythrin at 490 nm results in emission at 575 nm (compare panels a and c, in Fig. 2).

In fluorescence labeling, scattering and autofluorescence from the target being analyzed contribute background at the detection wavelength and set a limit to the sensitivity of a fluorescence assay. Autofluorescence in biological systems arises from such ubiquitous components as porphyrins and flavins. Such autofluorescence is characterized by a small Stokes shift. Consequently, the large Stokes shift of a phycobiliprotein conjugate used as a fluorescent tag allows detection at longer wavelengths where most of the aut-

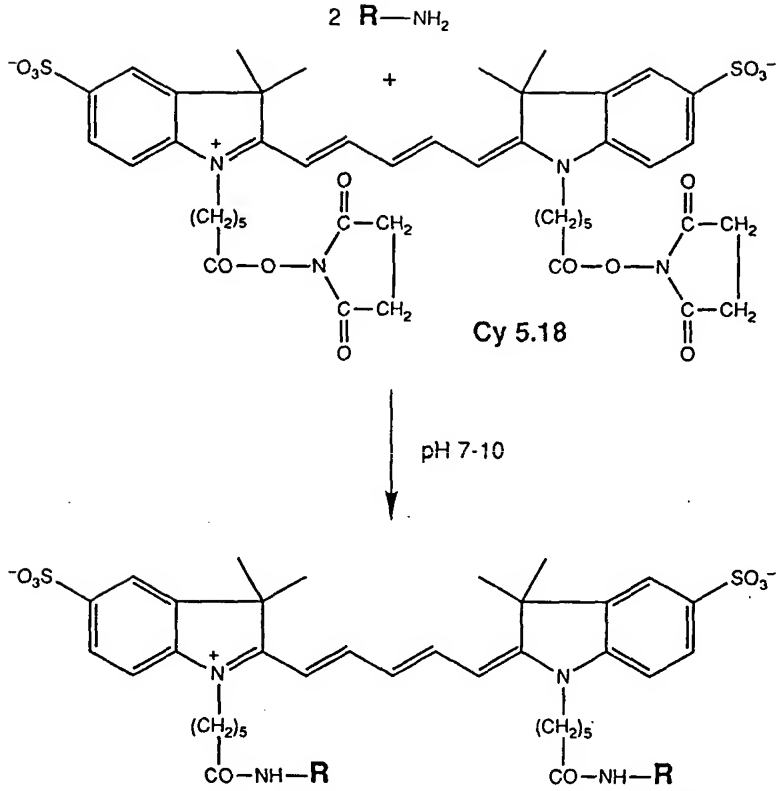

Fig. 3. Reaction of the succinimidyl ester derivative of sulfoindocyanine dye Cy5.18 with an alkylamine. The formation of protein-Cy5.18 conjugates is expected to proceed largely through this type of reaction with the ε- amino groups of lysyl residues. Spectroscopic data for a derivative of Cy5.18 with a low molecular weight alkylamine in phosphate buffered saline: ε_M (650 nm) is 2.5×10^5 M^{-1} cm^{-1} and Φ_F is 0.27 (λ^F_{max} 667 nm). (Data from Mujumdar *et al.*, 1993.)

ofluorescence contributions are eliminated along with scattering.

The availability of phycobiliproteins that absorb optimally in the red region of the spectrum is particularly advantageous. At wavelengths above 600 nm, because the absorption bands for the biomolecules which give rise to autofluorescence lie at shorter wavelengths, autofluorescence from cells falls off sharply. Consequently, the signal-to-noise ratio attainable with allophycocyanin conjugates is very high (Loken *et al.*, 1987; Fuchs *et al.*, 1988). Allophycocyanin is efficiently excited by the He-Ne laser at 633 nm, and its fluorescence emission maximum lies at 660 nm.

Intermolecular energy transfer

The Stokes shift can be greatly increased by exploiting intermolecular energy transfer, further enhancing the advantages noted above. For example, Glazer and Stryer (1983) showed that in a B-phycoerythrin-allophycocyanin conjugate, the energy absorbed by phycoerythrin was transferred with an efficiency of 90% to allophycocyanin. Excitation of the conjugate at 488 nm led to maximum fluorescence emission at 660 nm, a 172 nm Stokes shift.

Such intermolecular energy transfer has also been exploited by synthesizing conjugates of R-phycoerythrin with simple cyanine dyes, such as Cy5.18 (Fig. 3; Mujumdar *et al.*, 1993). In conjugates containing three molecules (or more) of Cy5.18 per phycoerythrin hexamer, energy transfer to the cyanine dye, with an emission maximum at 667 nm, proceeds with an efficiency exceeding 90% (see Fig. 3, panel c; Waggoner *et al.*, 1993).

Conjugates that utilize energy transfer have several advantages. First, they exploit the high extinction coefficient of the phycobiliprotein (energy donor) at the excitation wavelength. They allow simultaneous excitation of several different tags at the same

wavelength. This simultaneous excitation is important for multiparameter analyses with a single laser. For example, a fluorescein-conjugate (λ^F_{max} 530 nm), a R-phycoerythrin conjugate (λ^F_{max} 575 nm), and a R-phycoerythrin-Cy5.18 conjugate (λ^F_{max} 667 nm) can all be efficiently excited at the 488 nm argon ion laser line and their emissions detected in parallel in difierent channels at the appropriate wavelengths.

Other applications of phycobiliproteins

Histochemistry

The features which make the phycobiliproteins valuable as tags in cell analyses are equally important in the use of phycobiliprotein conjugates in histochemical applications. Combinations of appropriate phycobiliprotein conjugates and dye-conjugates specific for different markers allow simultaneous localization of several antigens in a single tissue section by fluorescence microscopy (e.g. Hermiston *et al.*, 1992). The exceptionally high molar absorbance of phycoerythrin conjugates has been exploited to detect low copy number antigens on cell surfaces by confocal laser scanning fluorescence microscopy. For example, the binding of epidermal growth factor (EGF) to different cell types was explored by using biotinylated EGF complexed with phycoerythrin-labeled anti-biotin antibody. As few as 10,000 EGF receptors per cell were detectable above background (Good *et al.*, 1992). The sensitivity of this procedure is comparable to that achievable with radioactivity and offers the advantage that optical sections of the labeled cell or tissue can be processed into three-dimensional images and used to determine the spatial distribution of the target antigens.

Assay of reactive oxygen species

Highly sensitive phycoerythrin fluorescence-dependent assays for peroxy radicals and for hydroxyl radicals generated at sites of metal ion chelation have been developed (Glazer 1988, 1990). These assays allow rapid screening for biologically relevant compounds that may protect cells against free radical damage as well as quantitation of the total amount of rapidly reacting free-radical scavengers in biological fluids (DeLange & Glazer, 1989).

Future prospects

Novel phycoerythrins

Certain marine unicellular cyanobacteria found in the open ocean (Waterbury *et al.*, 1986; Olson *et al.*, 1990) have phycoerythrins, rich in phycourobilins, that carry at least 37 bilin prosthetic groups (Ong & Glazer, 1991; Swanson *et al.*, 1991; Wilbanks & Glazer, 1993). These phycoerythrins have the highest molar absorption coefficients, at 490 nm, known for any phycobiliprotein. These potentially valuable proteins have been extensively characterized (Ong *et al.*, 1984; Ong & Glazer, 1991; Swanson *et al.*, 1991; Wilbanks & Glazer, 1993). However, until recently, the marine unicellular cyanobacteria had not been cultured on a large scale and the application of their phycobiliproteins as fluorescent tags has yet to be explored.

Semisynthetic phycobiliproteins

The bilins are attached to the phycobiliproteins through thioether bonds (Fig. 1). The information available to date on the enzymology of bilin attachment and on the factors that control the choice between isomeric bilins (Fig. 1) for attachment at a particular cysteinyl residue is very limited.

The biochemistry of bilin attachment has been defined only for the α-subunit of C-phycocyanin. This subunit carries a single bilin, a phycocyanobilin, at Cys-84. Two proteins, CpcE and CpcF, encoded by genes *cpcE* and *cpcF* in the C-phycocyanin operon in cyanobacteria, constitute a phycocyanin α-subunit phycocyanobilin lyase and catalyze the addition of phycocyanobilin to the apo- α-subunit both in vivo (Zhou *et al.*, 1992; Swanson *et al.*, 1992) and *in vitro* (Fairchild *et al.*, 1992).

The phycocyanin α-subunit phycocyanobilin lyase also catalyzes the *in vitro* addition of phycoerythrobilin to recombinant C-phycocyanin apo-α-subunit (Fairchild, 1993). A new type of phycocyanin subunit can thus be produced *in vitro*. Recombinant apo-subunits of various phycobiliproteins can be readily produced in *E. coli* or yeast. The prospects appear good of generating phycobiliproteins with predetermined bilin compositions suited to particular applications.

Acknowledgements

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